Reaction Mechanism of Protocatechuate 3,4-Dioxygenase

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Iron Dioxygenase, Non-Heme Iron, Reaction Mechanism

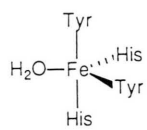
We have observed that high SOD-like function (decomposition of superoxide anion) was observed for several iron(III) compounds with tripodal ligands and several oxovanadium(IV) compounds, and also that these compounds exhibit high catalytic activity for oxidative cleavage of 3,5-di-*tert*-butylcatechol in non-donating solvents such as dichloromethane or nitromethane. These are suggesting that the same reaction intermediate exists in reaction mixtures of both the SOD-like and catecholase-like functions of these compounds. Based on these facts, we have proposed a new reaction mechanism for the oxidative cleavage of catechol catalyzed by the native non-heme iron dioxygenases; this includes formation of an iron(III)-peroxide adduct as a reaction intermediate, which exhibits electrophilic nature.

Introduction

A new type of enzyme with distinctly different properties from "oxidases" and "dehydrogenases" was discovered in 1955 by O. Hayaishi and H. S. Mason working independently with their associates [1, 2]. The enzyme, now referred to as "oxygenase", catalyzes the incorporation of either one or two atoms of molecular oxygen into an organic molecule. As results of extensive studies carried out during the following decade, the physiological and medical significance of this new type of enzyme involved in biological oxidation was established [3]. However, the intrinsic nature of the reaction mechanism, that is, why and how a dioxygen molecule is introduced into organic molecules, is not clear at present. In this article, we will focus on the reaction mechanism of protocatechuate 3,4-dioxygenase (hereafter abbreviated as 3,4-PCD), which is one of the non-heme iron dioxygenases.

3,4-PCD and Fe-SOD

3,4-PCD catalyzes the cleavage of protocatechuate (3,4-dihydroxobenzoate) to β-carboxy-*cis,cis*-muconic acid with incorporation of the element of dioxygen. The non-heme iron containing enzyme mediates the critical opening step in the pathway of biodegradation of many aromatic compounds, and it has been reported to be present in at least 10 bacteric strains spanning the aerobic genera [4]. X-ray crystallographic studies of 3,4-PCD from *Pseudomonas aeruginosa* at a resolution of 2.8 Å have shown the enzyme to be a dodecamer of two nonidentical subunits, *i.e.*, $(Fe^{3+})_{12}$ [5]. The active site lies at the interface between the α and β subunits with catalytic ferric ion coordinated by four residues from the subunit: Tyr 118, Tyr 147, His 160 und His 162. The iron coordination geometry forms an approximate trigonal bipyramid with Tyr 118 (β) and His 160 (β) located in the trigonal plane together with a coordinated solvent molecule as shown below.



Many mechanistic studies on this enzyme have been reported [6], but there is no report that relates the reaction mechanism of the enzyme with the unique coordination geometry around the iron atom.

In our previous paper [7], we have reported that the structure of the intermediate complexes with substrate and with inhibitor in 3,4-PCD should be different from each other, and assumed that it should be a 5-coordinate and a 6-coordinate for those with substrate and inhibitor, respectively, on the basis of the ESR spectra. Our assumption was supported by the recent EXAFS study of Que *et al.* [8]. We have proposed that the coordination of dioxygen molecule to the vacant lobe of the d_σ-orbital in the presence of monodentate catechol is

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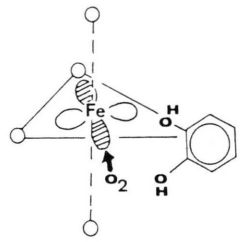
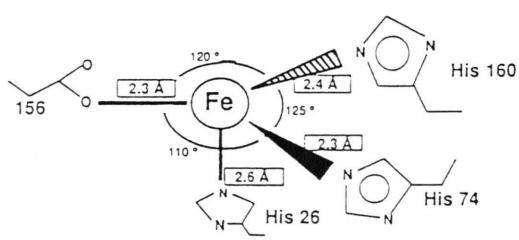


very important in the reaction of this enzyme (see the figure above) and showed that 6-coordinated Fe(III) complexes containing catechol as a bidentate ligand do not react with dioxygen molecule. This is partially consistent with the reaction mechanism proposed by Que *et al.* [9], but they did not explain the reason for the facile formation of the reaction intermediate. Thus, our model was the first report on the relationship between the reaction mechanism and the unique structure of iron atom in 3,4-PCD, and has demonstrated that the trigonal bipyramidal structure in 3,4-PCD is necessary for coordination and activation of dioxygen molecule.

In 1990, Stoddard *et al.* have determined the structure of Fe-containing SOD (superoxide dismutase) [10]. According to their results, it is clear that iron atom in this enzyme is coordinated by four ligand atoms, and the ferric coordination geometry forms an approximately trigonal pyramid (see the figure below), which is very similar to the case of 3,4-PCD. At present, there is no report that relates the structure and the SOD function of this enzyme. We have investigated the SOD-like function of many iron(III) compounds [11], and found that several iron(III) complexes with tripodal-like ligands such as (ntb), (pb2) or (tpa) exhibit high SOD-like function, whereas the SOD-like function of iron(III) compounds with tetradentate Schiff bases such as H_2 (salen) and H_2 (acen), Fe(salen)Cl or Fe(acen)Cl, is negligible (for the chemical structures of the ligands, see Fig. 1). On the basis of these facts, we have assumed that the SOD-like function of the iron(III) compounds with tripodal-like ligands appears as follows; at first the iron(III) complex is reduced to a iron(II) by superoxide anion, which is supported by the electrochemical data of these compounds, and the iron(II) species thus formed reacts with another superoxide anion, to yield an iron(III)-peroxide adduct, as shown below. The re-oxidation of the iron(II) species by superoxide anion, associated with formation of a Fe(III)-peroxide adduct, was confirmed by absorption and ESR spectroscopic studies on the compounds [11]. The re-oxidation of the reduced



Schematic illustration of Fe-SOD [10]

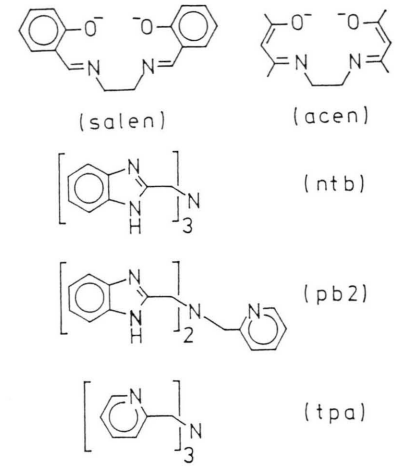


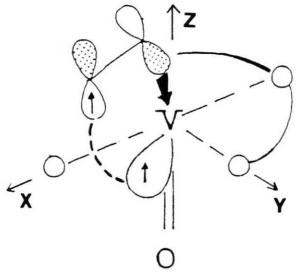
Fig. 1. Chemical structures of ligands cited in this paper.

$$Fe(III) + O_2^- \rightarrow Fe(II) + O_2$$

$$Fe(II) + O_2^- \rightarrow Fe(III)(O^-)(O^-)$$

Fe(II)-species did not proceed in the reaction between superoxide anion and metal compounds with tetradentate Schiff bases. Above facts are suggesting that the geometrical feature around the iron atom effects greatly on the re-oxidation step of a Fe(II) species by superoxide anion, and this seems to be consistent with the following experimental fact. We have observed that some oxovanadium(IV) complexes such as VO(salen) and VO(acen) show high SOD-like function, whereas no activity of SOD-like function was observed for

the compounds such as VO(saldpt) and VO(TTP) [12], where H_2(saldpt) and H_2(TTP) represent the Schiff base derived from salicylaldehyde and dipropylenetriamine, and α,β,γ,δ-tetra(p-tolyl)porphyrin, respectively. The electrochemical, absorption, and ESR spectroscopic data also revealed that VO(salen) and VO(acen) reacts with superoxide anion to yield a V(V)-peroxide adduct, but VO(saldpt) and VO(TTP) do not react with superoxide anion under the same experimental conditions. This is demonstrating that the concept of "two-point interaction" is important for the interpretation of above experimental results; that is, superoxide anion approaches to the vanadium atom through the coordination to the vacant position trans to V=O bond, and this superoxide anion accepts to electron from d_{xy}-orbital which contains one unpaired electron, to yield a V(V)-peroxide adduct. This leads to high SOD-like function of VO(salen) and VO(acen), and the "two-point interaction" is impossible for VO(saldpt) and VO(TTP), because all the four lobes of d_{xy}-orbital are screened by the ligand system in VO(TTP), and six coordination positions are occupied by the ligand system including the oxo oxygen atom in VO(saldpt). The same idea as described above may be applied for the elucidation of SOD-like function of iron(III) compounds with tripodal-like ligands.

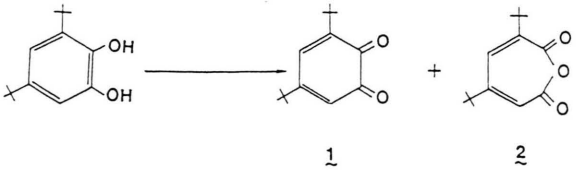


Schematic illustration of "two-point interaction" [12]

These are demonstrating that the SOD-like function of the model compounds is closely related with the formation of a peroxide adduct with higher oxidation state of a metal ion, and implying that the structure of the trigonal bipyramid in 3,4-PCD and the trigonal pyramid in Fe-SOD should be closely related with the facile formation of a peroxide adduct as a reaction intermediate.

Model Reaction of Catechol Dioxygenase

As the model reaction for catechol dioxygenase, 3,5-di-*tert*-butyl-catechol is widely used as a substrate. In this case, we can obtain quinone, **1,** and corresponding acid anhydride, **2,** as main products. In general, the presence of the metal compounds in the solution of this substrate, gives only quinone as a reaction product, and formation of acid anhydride, which is believed to be obtained through the oxygenation reaction, is negligible.

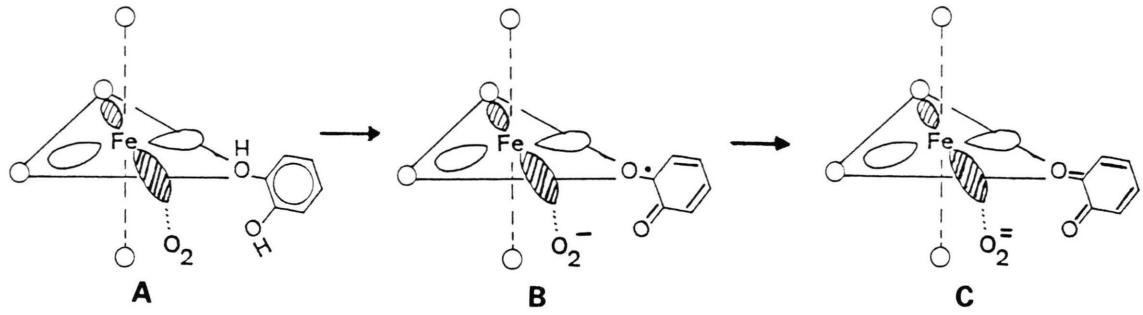


1 2

In 1982, Otsuka *et al.* [13] have reported that some oxovanadium(IV) compounds such as VO(salen) and $VO(acac)_2$ exhibit high catalytic activity for the formation of **2,** but the activity of VO(saldpt) and VO(TPP) is negligible, where H_2(TPP) denotes α,β,γ,δ-tetraphenylporphyrin. This clearly implies that SOD-like function of the oxovanadium(IV) compounds is closely related with its ability for the oxygenation of 3,5-di-*tert*-butylcatechol. Similar facts are also observed for the iron(III) compounds. We have observed that Fe(salen)Cl or $Fe_2O(salen)_2$ complexes do not show activity for the oxidative cleavage of the catechol, but the iron(III) complexes with tripodal-like ligands exhibit the activity for the oxygenation of catechol [14, 15]. This suggests that a complex formation with superoxide anion, and subsequent formation of a Fe(III)-peroxide adduct may play an important role in the catecholase-like function of the model systems.

New Mechanism of Catechol Dioxygenase

Now we would like to propose an alternative reaction mechanism for the 3,4-PCD. At first, we assume that the catechol approaches to iron atom at the corner of the trigonal (intermediate *A* as illustrated below), as pointed out in the previous section. A coordination of catechol to an iron atom stimulates the coordination of dioxygen molecule to the vacant lobe of the d-orbital because of the increased electron density on the metal ion due



to the coordination of catechol. Then the electron of catechol transfers to dioxygen through a d-orbital, forming a semiquinone-Fe(III)-O_2-(intermediate *B*). This Fe(III)-superoxide complex may change to a iron(III)-peroxide adduct by accepting the electron from the semiquinone through a d-orbital, to yield a quinone-Fe(III)-peroxide (intermediate *C*). The unique geometry around the iron atom in 3,4-PCD should allow easily these changes. As previously pointed out [16, 17], the peroxide ion in the intermediate *C* contains some degree of singlet oxygen ($^1\Delta_g$) character, *i.e.*, exhibits electrophilicity because of the presence of unoccupied orbital comprised of d-orbital and π^*-orbital of peroxide ion. This activated peroxide adduct attacks to the quinone through HOMO-LUMO interaction, giving a oxygenated product, where HOMO and LUMO denote highest occupied and lowest unoccupied molecular orbital, respectively. Our model may be also supported by the recent report by Hianchini *et al.* [18]; they isolated the semiquinone-metal peroxide adduct as crystal.

High catecholase-like function observed for several oxovanadium(IV) compounds may be rationalized by the similar way; in this case the lobes of d_{xy}-orbital which are not screened by the ligand system should play an important role in interaction with dioxygen, and in activating it.

Lipoxygenases are well known one of the non-heme dioxygenases [19]. At present although the mechanism of oxygen activation in this enzymatic reaction is not known [20], it seems likely that the reaction mechanism proposed for 3,4-PCD in this article may be applied to the lipoxygenases, because the assumed structure for a peroxy derivative in lipoxygenase [21] is very similar to that assumed in this paper, and also participation of activated oxygen containing singlet oxygen character has been pointed out in the peroxidation reaction of linolenic acid [22, 23].

Formation Mechanism of Fe(III)-peroxide Adduct in the Model Systems

In the previous section, the formation of a Fe(III)-peroxide is assumed to be the most important step in the oxygenation reaction in non-heme dioxygenases. In the case of 3,4-PCD, two electrons may be provided from the substrate; catechol $\rightarrow 2e^- +$ quinone. The formation of the oxygenated iron(III) species is sometimes observed in the model systems. We have recently observed that Fe(III)-edta/ascorbic acid system exhibits high activity for degradation of DNA, whereas its ability of Fe(III)-detapac/ascorbic acid system is negligible, where H_5 (detapac) represents diethylentriaminepentaacetic acid.

As illustrated in Fig. 2, the electrochemical properties of Fe(III)-edta$^-$ and Fe(III)-detapac^{2-} are similar to each other under an atmosphere of nitrogen; Fe(III) state is reduced to Fe(II) state at -0.18 and -0.22 V (*vs.* SSCE, for SSCE see Fig. 2) for Fe(III)-edta$^-$ and Fe(III)-detapac^{2-}, respectively. However, the presence of dioxygen molecule in solution gave drastic change in cyclic voltammograms (CV) of Fe(III)-edta complex. As shown in Fig. 2, reduction wave of dioxygen is observed -0.42 V (*vs.* SSCE) under our experimental conditions. The CV property of Fe(III) ion of Fe(III)-detapac^{2-} complex is nearly independent on the presence of dioxygen molecule; both of the reduction waves, Fe(III) $\rightarrow$ Fe(II) and $O_2 \rightarrow O_2$ are observed separately, and no increase or decrease of current for reduction of oxidation step was observed in the Fe(III) $\rightleftharpoons$ Fe(II) process. On the other hand, in the case of Fe(III)-edta complex the CV exhibited increased current for the reduction

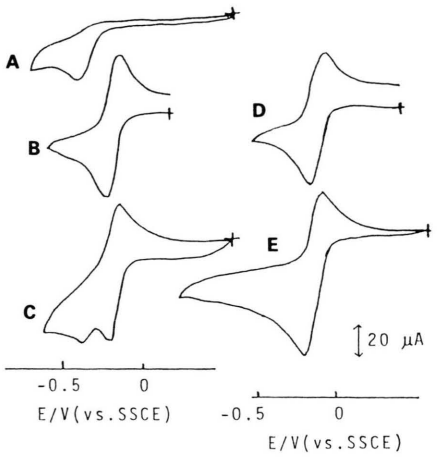
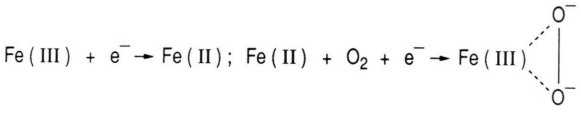


Fig. 2. Cyclic voltammograms (CV) of the compounds. The electrochemical measurements were made as follows: in 0.5 M Na_2SO_4, 25 °C, 0.004 M metal complex, glassy carbon electrode (commercially obtained), scan speed, 0.1 V/s, and the potential was referenced to (SSCE), saturated sodium chloride electrode. The CV data of O_2 and metal complexes in the presence of O_2 were done after bubbling O_2 gas through the solution for 20 min. A: O_2 (saturated); B: Fe(III)-detapac^{2-} (under N_2); C: Fe(III)-detapac^{2-} (under O_2); D: Fe(III)-edta$^-$ (under N_2); E: Fe(III)-edta$^-$ (under O_2).

wave of Fe(III) → Fe(II) (−0.18 V *vs.* SSCE) relative to that observed under anaerobic conditions. This increase suggests that Fe(II)-edta^{2-}, once formed, can combine with another dioxygen molecule and undergo further reduction at the electrode surface, leading to formation of a Fe(III)-peroxide. The analogous observations have been made for reduced Fe and Mn complexes [24, 25]. We also found that the electrochemically reduced, oxygenated Fe(II)-edta complex exhibits high ability for DNA degradation (the solution containing DNA and Fe(III)-edta complex was electrolyzed at −0.20 V (*vs.* SSCE) under constant flow of air), however, the ability of Fe(III)-detapac^{2-} complex for DNA degradation is negligible under the same experimental conditions as described for Fe(III)-edta$^-$ complex.

The foregoing data are all consistent with the activation mechanism involving initial reduction of Fe(III)-edta$^-$, followed by simultaneous binding with O_2 and electron, giving an iron(III)-peroxide adduct (see the figure below), which may be a



true active species for DNA degradation [26]. This is also indicating that oxygenated species easily forms in the presence of dioxygen and reductants such as ascorbic acid or electron form the electrode if the geometric factor around the iron atom is favorable, and this elucidates the high ability of Fe(III)-edta/ascorbic acid system for DNA degradation, and also supports the reaction mechanism proposed for 3,4-PCD in this article.

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